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A Mechanistic Study of Proapoptotic Daxx-Par4 Axis in Prostate Cancer

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## Introduction

DAPK1, the founding member of the DAPK family (DAPK1-3), is a  $\text{Ca}^{2+}$ /calmodulin Ser/Thr kinase that promotes cell death [1]. DAPK1 functions as a tumor suppressor gene, and its expression is lost in many cancer types [2]. A positive connection between DAPK1 and autophagy has been established in mammalian cells and *C. elegans* [3-6]. DAPK1 and DAPK3 are both target genes of the NF- $\kappa$ B member, RelB, and are potently repressed via epigenetic mechanisms by the transcriptional repressor Daxx, which interacts with RelB [7, 8]. Since DAPK1 triggers autophagy [5, 6], suppression of DAPK1 by Daxx might block autophagic cell death. Suppression of autophagy is often found in malignant transformation [9-18]. Recent data indicate that autophagy acts as a tumor suppression mechanism in ALVA-31 prostate carcinoma cells [19]. In the current study, I used retroviral-mediated shRNA to silence *Dapk1*, *Dapk3*, and both *Daxx* and *Dapk1* in the prostate cancer cell line ALVA-31. Through preliminary subcutaneous xenograft experiments (Figs. 1 & 2), I have determined that (1) *Daxx* is required for tumor growth, because *Daxx* depletion in ALVA-31 cells correlates with smaller tumors; and (2) DAPKs act as tumor suppressors, because tumor growth rate is faster for DAPK3 knock-down (K/D) and DAXX/DAK1 double K/D groups.

*Daxx* is a transcriptional repressor, binding partner of tumor suppressor PML, and a component of PML nuclear bodies ("PODs") [7, 8, 20, 21]. Mammalian *Daxx* (Fig. 4A) is poorly characterized at the structural level, although it bears limited sequence similarity to the transcriptional co-repressor Sin3a [22]. The *Daxx*-null genotype is an embryonic lethal condition [23]. The cause of death of *Daxx*<sup>-/-</sup> embryos is global apoptosis, suggesting an anti-apoptotic role for the *Daxx* protein during development [23]. *Daxx* physically interacts with transcription factor RelB [7]. RelB (Fig. 4B) is an unusual member of the NF- $\kappa$ B transcription factor family in that it acts as both a transcriptional activator and as a repressor of NF- $\kappa$ B-dependent gene expression [8, 24, 25]. We have proposed that RelB links *Daxx*'s transcriptional and cell death activities. Relevant to this aim, we plan to solve the X-ray crystal structure of *Daxx* bound to RelB with the aim of understanding of the functional significance of *Daxx*-RelB interaction. *Daxx* (Fig. 4A) physically interacts with RelB (Fig. 4B), but no other members of the Rel family [7]. As we reported in the last progress report, we mapped the *Daxx* interaction domain in RelB by co-precipitation studies with proteins transiently-overexpressed in human embryonic kidney (HEK) 293T cells, and this maps to DID-1 region that interrupts the RelB DNA-binding domain. This year's efforts established that (1) the interaction between *Daxx* and RelB is direct (using purified proteins in pull-down experiments); and (2) *Daxx* amino acids 190-400 are necessary and sufficient for *Daxx* interaction with RelB (Fig. 3). With this information as a starting point for our structure project, various relevant *Daxx* and RelB domains have been expressed in large quantities and purified, and will next be used for co-crystallization of the interacting fragments and determination of their crystal structures (Fig. 5). We will then exploit the structural information to probe *Daxx*'s functions.

Several anti-apoptotic genes have been discovered that are over-expressed in hormone-refractory prostate cancers [26]. Several lines of evidence indicate that in a cancer context, like in development, *Daxx* may have a pro-survival role. In prostate tissues, *Daxx* staining intensity is stronger in areas surrounding tumor glands compared to areas surrounding normal glands [27]. In prostate cells, *Daxx* levels are increased in various human prostate cancer (PCa) cell lines, relative to nontumorigenic human prostatic epithelial lines [19]. Conversely, in cells and tissues, DAPK1 is downregulated in cancer contexts [9, 19]. In the current study, an inverse correlation was observed between *Daxx* and DAPK1 mRNA expression in a diverse collection of human tumor cell lines and tumor specimens (Fig. 6), suggesting that *Daxx*'s role as a transcriptional repressor of the DAPK1 gene is broadly relevant to tumor biology, and is not limited to PCa.

To summarize the significance of the proposal, our studies will unravel how the tumor suppressor/autophagy inducer DAPK1 works, how the transcriptional repressor Daxx, in conjunction with transcription factor RelB, affects its functions in vivo, and how we might summon the DAPK-Daxx axis into action against aggressive prostate cancers. Furthermore, our proposed experiments form a structure-function framework for understanding Daxx's functions in vivo. Armed with such information, it may be possible to design peptide inhibitors, delivering them into cells via membrane penetrating sequences, so as to suppress Daxx's tumorigenic actions by blocking RelB binding sites. This would result in restored activity of tumor suppressor DAPK1. Restoring the activity of endogenous tumor suppressors is an attractive strategy for controlling malignancy, because it taps into natural pathways for self-defense against cancer.

## Body

### Results

#### **Daxx depletion in ALVA-31 cells correlates with smaller tumors and increased autophagy markers in subcutaneous xenografts**

In the *Conclusion* section of last year's progress report (page 22), we reported that "We are now extending Daxx studies from cell culture to in vivo mouse cancer models, exploring the mechanisms for inhibiting prostate tumor growth in animals using human prostate cancer xenografts in immunocompromised mice". To that end, in preliminary experiments to investigate the role of Daxx in prostate tumorigenesis, we utilized an athymic nude mouse model. Each mouse was injected subcutaneously with  $10^6$  human ALVA-31 Daxx knock-down (K/D) or ALVA-31 control shRNA (CNTL) prostate cancer cells that had previously been generated (see last year's progress report, pages 3, 5, and 11). The tumors were grown for 5 weeks, monitoring tumor volume by external calipers every few days and determining the wet weights of tumors at termination of the experiments. Three independent experiments were performed. We found that the size of subcutaneous ALVA-31 prostate carcinoma cell xenograft tumors in nude mice was significantly smaller with cells in which Daxx expression was stably reduced by shRNA (Daxx K/D cells) than with control ALVA-31 cells (**Fig. 1A**). Moreover, the Daxx-depleted tumors exhibited elevated levels of DAPK1 protein, and also an increase in the autophagy markers Beclin-1 and LC3, as determined via IHC (**Fig. 1B**). In conclusion, Daxx promotes tumorigenesis in a prostate cancer xenograft model, possibly by repressing DAPK1 and other autophagy inducers.

#### **Tumor growth rate is faster for DAPK3 K/D and DAXX/DAPK1 double K/D groups, arguing that DAPKs are acting as tumor suppressors**

To test the effect of autophagy inducers, such as DAPKs, on prostate tumorigenesis, using shRNA, I stably depleted (90% knockdown efficiency) DAPK1 and DAPK3 individually in control and DAXX K/D ALVA-31 cells (**Fig. 2A**). I then determined their effects on tumorigenesis using a mouse subcutaneous xenograft model. Three nude mice per group were injected each with  $10^6$  cells. The tumor growth kinetics from these preliminary data show that the tumor growth rate is faster for the DAPK3 K/D and DAXX/DAPK1 double K/D groups relative to the control shRNA group (**Fig. 2B**), arguing that DAPKs are acting as tumor suppressors. Incidentally, mice injected with DAPK3 shRNA cells (and the DAXX/DAPK1 double K/D – not shown) manifest more aggressive tumors (**Fig. 2C**), and we are currently performing histopathological studies to determine if the respective tumors have metastasized. While this particular experiment involving DAPKs was not part of the original Statement of Work, we felt it was important to incorporate it into the Tasks, given our finding -- which emanated from Task 2 -- that DAPKs are the main targets of Daxx's suppression (last progress report, Figs. 2 & 3, pages 8, 12, and 13). These previous findings provided important clues to Daxx's involvement in autophagy inhibition via suppression of autophagy modulators, such as DAPK1. We are, accordingly, planning to investigate the mechanisms of DAPK1-DAPK3-Daxx axis in autophagy and tumorigenesis by performing a larger scale xenograft study modeled after the one described above.

#### **The interaction between Daxx and RelB is direct, and involves the N-, rather than C-, terminus of Daxx**

Relevant to Task 1, we plan to solve the X-ray crystal structure of Daxx bound to RelB with the aim of understanding of the functional significance of Daxx-RelB interaction. Daxx physically interacts with RelB, but no other members of the Rel/NFκB family [7]. To this end, last year we mapped the Daxx interaction domain in RelB by coprecipitation studies with proteins transiently-overexpressed in human embryonic kidney (HEK) 293T cells, and this maps to DID-I region lying in the DNA binding domain (Task 1 and last progress report, pages 3, 4, 8, 9, 15, and 16). Through GST pull-down assays, using seven Daxx-GST fragments (**Fig. 3A**) and the His-tagged N-terminal RelB (containing the DID domain), I have now determined that the Daxx N-terminal 400 amino acids (in particular aa 190-400, containing the second coiled coil region) are necessary and sufficient for Daxx interaction with RelB, which is direct (**Fig. 3C**). Specifically, the N-terminal Daxx fragment, which contains two coiled coiled regions, interacts strongly with RelB (**Figs. 3C & 4A**). With this information as a starting point for our structure project, various relevant Daxx and RelB domains have been expressed in large quantities in *E. coli* and purified (**Fig. 3B**). Thrombin cleavage of the GST- or His-tag from the purified proteins has been completed, and crystallization and structure solution steps will follow (**Fig. 5**). We will then exploit the structural information to probe Daxx's functions, in accordance with the guidelines of Task 1.

### **Human malignancies display an inverse correlation between Daxx and Dapk1 mRNA expression**

Dapk1 is an autophagy-inducing kinase [5, 6], making it a strong candidate to explain the effects of Daxx on autophagy. Previous studies of human tumor cell lines in which Daxx levels were manipulated by gene transfection [7] or shRNA [19], as well as previous comparisons of *daxx*<sup>+/+</sup> and *daxx*<sup>-/-</sup> MEFs [8], have shown that Daxx functions as a transcriptional repressor of the gene encoding Dapk1 (previous year's progress report, pages 3, 12, and 13, corresponding to Task 2). Consequently, in tumor cell populations when this regulatory circuitry is engaged, one should observe inverse patterns of the Daxx and Dapk1 mRNA expression. To explore how pervasive the Daxx/Dapk1 axis is in human malignancy, we performed linear regression analysis of publicly available Daxx and Dapk1 mRNA expression data for the NCI60 panel of human cancer cell lines and the U95 database of human tumor tissue samples (<http://biogps.gnf.org/>). Strikingly, we found a statistically significant inverse correlation between the Dapk1 and Daxx mRNA expression levels in these tumor cell lines and tumor samples derived from diverse types of human cancers (**Fig. 6**). Notably, the inverse patterns of Dapk1 and Daxx mRNA expression were observed in both established cancer cell lines (**Fig. 6, A & B**) and tumor tissue samples (**Fig. 6, C & D**). These results imply that Daxx/Dapk1 regulatory axis broadly applies across a spectrum of human malignancies and does not represent an artifact of culturing cancer cells in vitro. These preliminary data, coupled with evidence for involvement of DAPK1 in cancer and in autophagy, suggest that Daxx regulates autophagy, at least in part, by repressing DAPK1 expression, which thereby reduces Beclin-1 phosphorylation [6], and suppresses autophagy.

### **Experimental Procedures**

#### ***Cell culture and recombinant lentivirus transduction***

Prostate cancer cell line ALVA-31 was maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium, containing 10% bovine calf serum, BCS (Hyclone), and 1% penicillin/streptomycin plus L-glutamine. For the generation of a stable Dapk1, Dapk3, or Daxx/Dapk1 (double) knock-down (K/D) PCa lines, recombinant lentiviruses targeting Dapk1, Dapk3, or Daxx (constructed in lentiviral backbone vector pLKO.1-puro) were purchased from

Sigma. A non-specific control virus was also purchased (SHC002V: MISSION® Non-Target shRNA Control Transduction Particles). When the ALVA-31 cells reached 70-80% confluence, they were infected (MOI=10) with the relevant shRNA or the nonspecific control shRNA viruses. Hexadimethrine bromide (polybrene, Sigma, Cat # AL-118), at a concentration of 8 ug/ml, was added at the time of infection to enhance infection efficiency. After 24 hr, the medium was changed and replaced with the puromycin-containing medium (Sigma, Cat # P9620; 2 µg/ml). Cells were cultured for ~ 3 weeks in puromycin-containing medium, before performing immunoblotting analyses (see below).

### ***Antibodies and immunoblotting***

Protein lysates were prepared by suspending cells in 1 ml of freshly-prepared lysis buffer (Tris, pH 7.4, 50 mM; NaCl, 150 mM; EDTA, 20 mM; NP-40, 0.5%; PMSF, 1 mM; and DTT, 1 mM), which contained protease inhibitors (leupeptin, 20 µg/ml and aprotinin, 20 µg/ml) and phosphatase inhibitors (NaF, 50 mM and Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM). Following centrifugation at 14x10<sup>3</sup> rpm for 10 minutes at 4°C, the protein concentration of the supernatants was determined by the Bradford assay-based Bio-Rad method (Bio-Rad DC Protein Assay, Catalog # 500-0111). Aliquots of cell lysates, normalized for total protein content, were fractionated by SDS-PAGE, and transferred to nitrocellulose blotting membranes (BA85 Protran, 0.45 µm, Whatman, Catalog # 10401196). The following antibodies were used for immunoblotting: rabbit anti-Daxx (Santa Cruz Biotechnology); rabbit anti-Dapk1 (Sigma); rabbit anti-Dapk3 (Sigma); and mouse anti-β-actin (Sigma). Immunoblotting was performed using the Odyssey Imager (LI-COR Biosciences). Data were quantified by scanning densitometry analysis of scanned gels, using *Scion Image* software.

### ***In vivo xenograft models***

For the subcutaneous xenograft model, the athymic nude male mice were purchased from Charles River Laboratories. Ten million cells were injected subcutaneously into the right flank of nine week-old animals. Tumor size was measured bi-weekly using external calipers. Mice were monitored daily for any sign of illness. Tumors were harvested 4-6 weeks post-injections, and their respective weights (g) determined immediately post-excision. Statistical analyses were performed using Student's t-test, Fisher's exact test, and chi-square test. Excised tumors were processed for immunohistochemical analyses (IHC). Tumors were submerged in Z-Fix (Anatech, Ltd., Cat # 174) solution for 4 days and then processed as described below.

### ***Immunohistochemistry***

Tumor tissues were immediately excised from sacrificed mice, fixed in zinc-buffered formalin (Z-fix; Anatech, Ltd.), and embedded in paraffin. Dewaxed tissue sections (4.0–5.0 µm) were immunostained using rabbit polyclonal antibodies to Beclin-1, Daxx (Santa Cruz Biotechnology, Inc., CA, USA), Dapk1 (Abgent Inc., San Diego, CA), as well rabbit monoclonal antibodies to LC3 (Abgent). Application of the primary antibody was followed by incubation with goat anti-rabbit polymer-based EnVision-HRP-enzyme conjugate (DakoCytomation). Hematoxylin or Nuclear Red (DakoCytomation) was used as counterstain.

### ***Protein expression, purification, and GST pull-down assays***

Seven Daxx-GST constructs were obtained from T. G. Hofmann (Heidelberg, Germany), and a purified RelB-His fragment (aa 1-400) was obtained from G. Ghosh (UC San Diego). Expression of Daxx-GST fragments was performed using *E. coli*, strain BL21. Purification of GST-fusion Daxx fragments was done using glutathione-agarose beads (Cat # G4510, Sigma). Purified Daxx (GST-tagged) and RelB (His-tagged) were subjected to GST pull-down assays, following a protocol provided by G. Ghosh (UC San Diego), to (1) determine whether the interaction between Daxx and RelB is direct, and (2) identify the Daxx region interacting with RelB. In preparation for co-crystallization studies, the tag (GST or His) was subsequently cleaved from purified Daxx or RelB, respectively, using a thrombin cleavage kit from Sigma (Cat # RECOMT).

### ***Analysis of Dapk1 and Daxx expression patterns in human cancer***

RNA expression data available in the <http://biogps.gnf.org/> were used to correlate Daxx and Dapk1 expression. Raw expression values were normalized using global normalization method to the average mRNA expression level of individual genes across the data set, log 10 transformed, and screened for discordant patterns of *Dapk1* and *Daxx* mRNA expression in individual samples. Samples were ranked in descending order of either *Dapk1* or *Daxx* normalized expression values and interrogated using linear regression analysis. In the microarrays where the multiple probe sets are assigned to the same gene, the analytical protocols were performed for at least two *Dapk1/Daxx* probe set combinations as well as the average normalized gene expression values and consensus results are reported for each data set. Two-tailed p values were computed for corresponding correlation coefficients using GraphPad 4.0 statistical analysis software package. G. Glinsky, at the Ordway Research Institute in Albany, NY, helped with this statistical analysis.

### **Figure Legends**

**Figure 1. *Daxx* depletion in ALVA-31 cells correlates with smaller tumors and increased autophagy markers in subcutaneous xenografts.** (A) The effect of Daxx knock-down on tumor growth was evaluated. Athymic mice were injected subcutaneously with  $5 \times 10^6$  ALVA-31 control vector (CNTL) or ALVA-31 Daxx shRNA (K/D) cells. Volumes and wet weights of tumors were measured at the termination of xenograft experiments (1 month). Results represent data from 3 experiments, involving a total of 18 mice injected with ALVA-31 control vector (CNTL) and 18 mice injected with ALVA-31 Daxx shRNA (K/D) cells. Statistical significance was determined by unpaired t-test ( $p < 0.05$ ). (B) Quantitative immunohistochemical analysis of protein markers was performed using excised tumor tissue corresponding to CNTL and Daxx K/D tumors. Tissue sections were stained using antibodies specific for Daxx, Dapk1, Beclin-1, and LC3. Statistical significance was assessed by unpaired t-test.

**Figure 2. The effect of DAPK1, DAPK3, DAXX, and DAXX/DAPK1 (double) knock-down on tumor growth kinetics in a subcutaneous xenograft model.** (A) Retrovirus-mediated shRNA gene transfer was used to stably silence DAXX, DAPK1, DAPK3, and both DAXX/DAPK1 in the hormone-refractory prostate cancer cell line ALVA-31. The knock-down level was quantified by RT-PCR for mRNA (not shown) and by immunoblotting for protein. The percentage reduction in each protein was estimated by scanning densitometry to be ~90% relative to untransfected or control (C) shRNA-infected cells. (B) Male athymic nude mice were injected in the right flank subcutaneously with  $10^6$  cells from each of the five cell groups indicated and tumor volumes

were measured over the course of one month using external calipers. Note that experiments typically require a group size of 6 to achieve statistical significance, and for this pilot study we employed a smaller group of mice (3 per group), hence we did not reach statistical significance. Nevertheless, tumor growth kinetics from these preliminary data show that tumor growth rate is faster for DAPK3 K/D and DAXX/DAPK1 double K/D groups, arguing that DAPKs are acting as tumor suppressors. Aliquots of various cell types used in injections were re-plated immediately following injections and determined to be fully viable. **(C)** Tumor appearance (one month post-injection) of two mice subcutaneously injected in the right flank with the indicated cells. Note that the mouse injected with DAPK3 shRNA cells manifests a much more aggressive tumor.

**Figure 3. *Daxx-RelB protein-protein interaction studies.*** **(A)** Daxx-GST fragments (obtained from T. Hofmann, Germany) were expressed in bacteria and purified **(B).** **(C)** GST-pull-down assays were performed using a denaturation/refolding protocol using purified bead-bound GST-Daxx fragments and purified His-tagged RelB containing Rel Homology Region (aa 1-400, obtained from G. Ghosh, UCSD). Note that the interaction between Daxx and RelB is direct (experiment performed > 5 times), and involves the N-, rather than C-, terminus of Daxx. The association is particularly strong between RelB and Daxx fragments “B”, “F, and “E” (red arrows indicate RelB pulled down with the respective Daxx fragment). Daxx fragment “B” contains two coiled coil regions. Crystallization steps will follow.

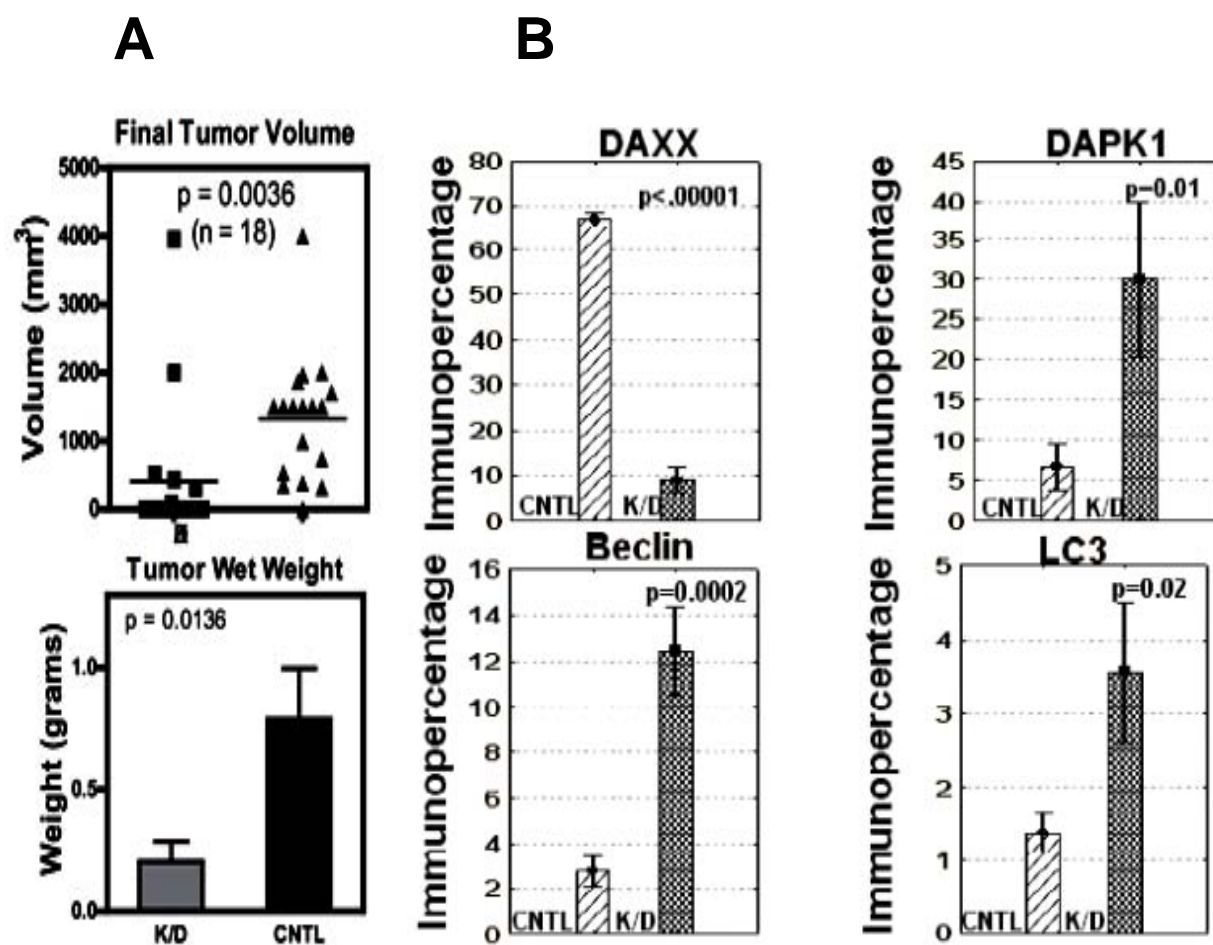
**Figure 4. *Schematic illustration of the domains of the human Daxx and RelB proteins.*** **(A)** Daxx is a 740-amino acid protein that contains several putative domains. The structural characteristics of Daxx are depicted by shaded boxes. Abbreviations: PAH, paired amphipathic helices; CC, coiled-coiled domain; D/E, acid-rich domain; NLS, nuclear localization signal; S/P/T, domain rich in serine, threonine, and proline; SIM, SUMO-interacting motif. The previously identified protein-protein interaction domains (aa 502–625 and aa 626–740) are depicted in black, whereas the newly identified RelB interaction domain, encompassing amino acids 190-400, is depicted in red. **(B)** RelB is a 558-amino acid protein, encompassing the following domains: Daxx Interaction Domain, DID, is composed of DID-I and DID-II regions and is conserved in ETS1, ETS2, FLI-1, PAX3, CENP-C, and RelB transcription factors [22, 41]. Human and mouse RelB share the same DID-I and are 89% identical in DID-II. The amino acid composition of each DID domain is shown, with underlined letters indicating conserved residues among all DID-containing transcription factors. The designation of the other domains is as follows: LZD, Leucine Zipper-like Domain; RHD, Rel Homology Domain; Ct, C-terminus. The previously identified (and reported in last year’s progress report) Daxx interaction domain is depicted in red.

**Figure 5. *The architecture of the Daxx protein.*** DAXX contains the recently characterized DHB (Daxx Helical Bundle) domain [28], along with another predicted helical region, whose structure we will attempt to solve in a complex with RelB. The uncharacterized region (depicted by a red double-headed arrow) binds RelB (Figs. 3 & 4). The other regions shown are intrinsically disordered (acidic (80% Glu/Asp residues), SPE (Ser/Pro/Glu rich), SPT (Ser/Pro/Thr rich), and SIMs (SUMO-interaction motifs)). The figure is adapted from Ref. 28.

**Figure 6. *Human malignancies display an inverse correlation between Daxx and Dapk1 mRNA expression.*** Linear regression analysis was performed using publicly available *Dapk1* and *Daxx* mRNA expression data for the NCI60 panel of human cancer cell lines **(A, B)** and the

U95 collection of human tumors (**C**, **D**). Individual samples were ranked in descending order of either *Dapk1* (**A**; **C**) (blue symbols) or *Daxx* (**B**; **D**) (red symbols) mRNA expression values and interrogated using linear regression analysis. Statistically significant inverse correlations between *Dapk1* and *Daxx* mRNA expression were observed for both NCI60 and U95 data sets (r and p values are provided in the figure).

Figure 1



# Figure 2

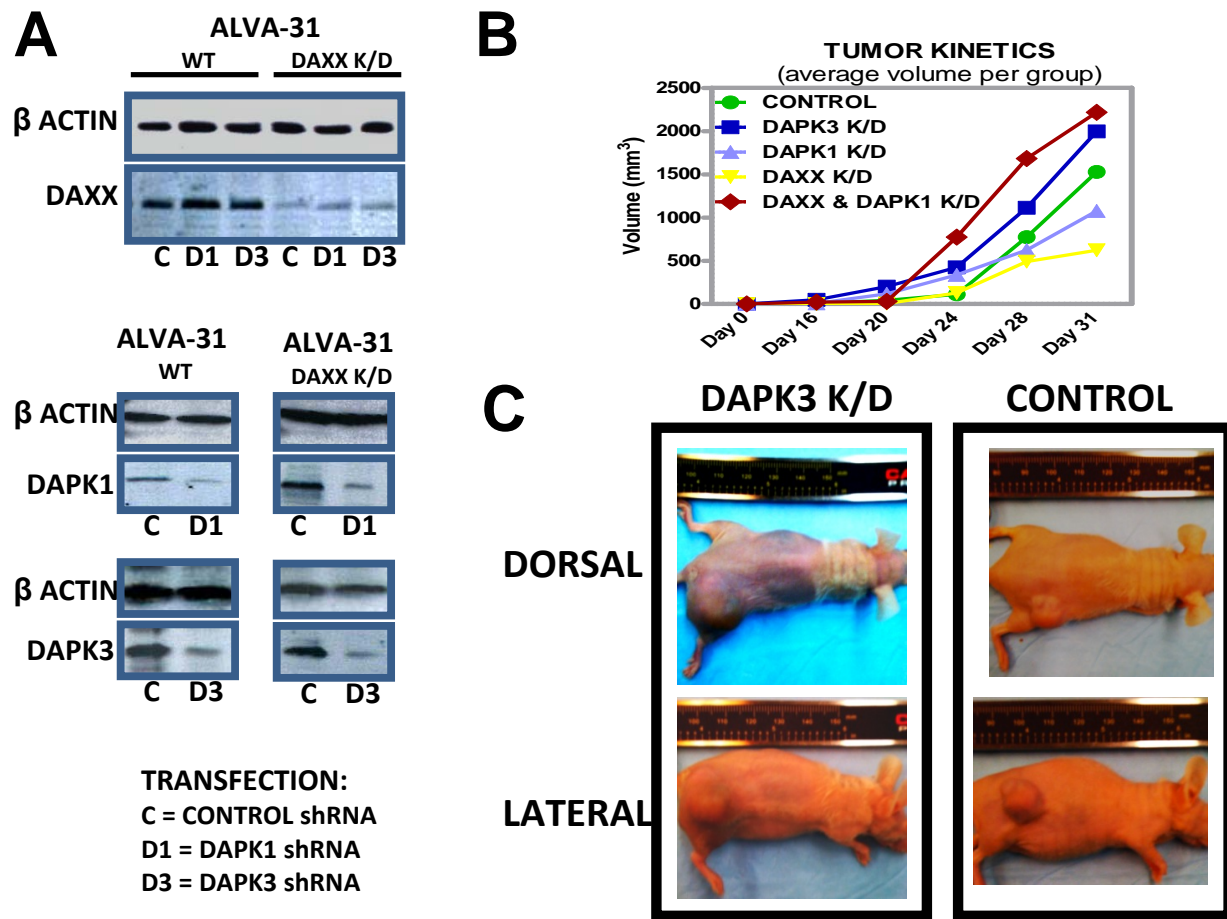


Figure 3

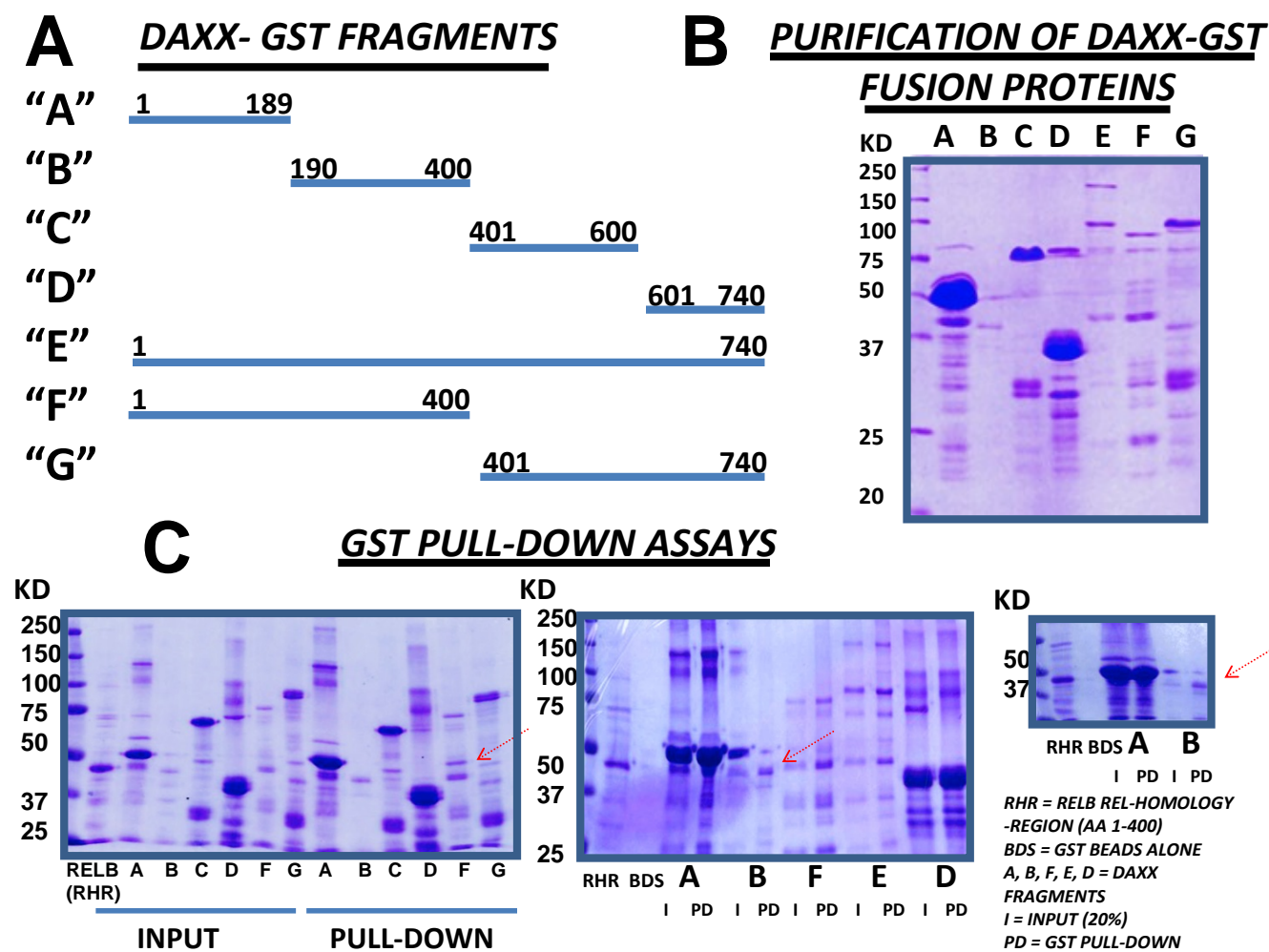


Figure 4

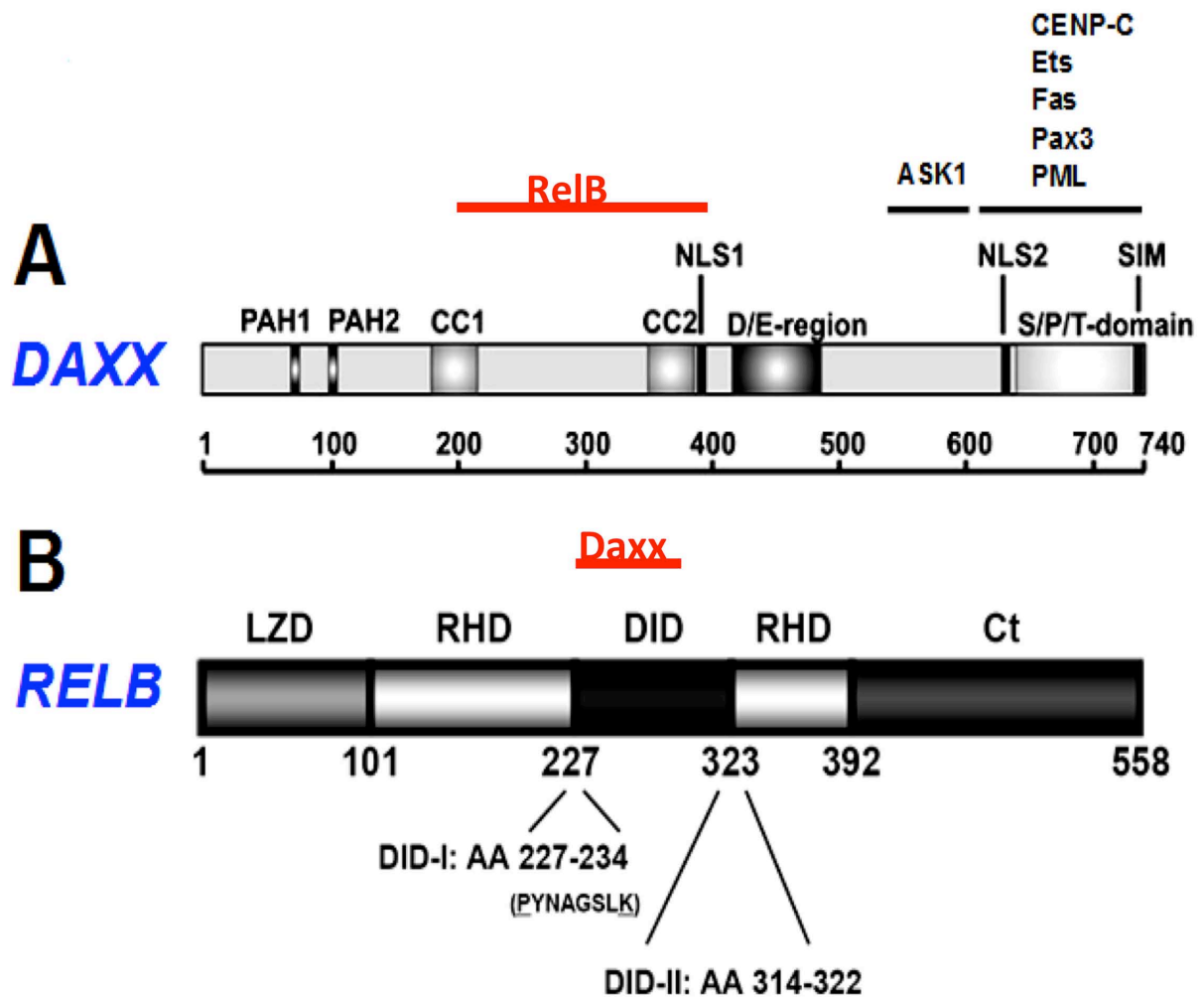


Figure 5

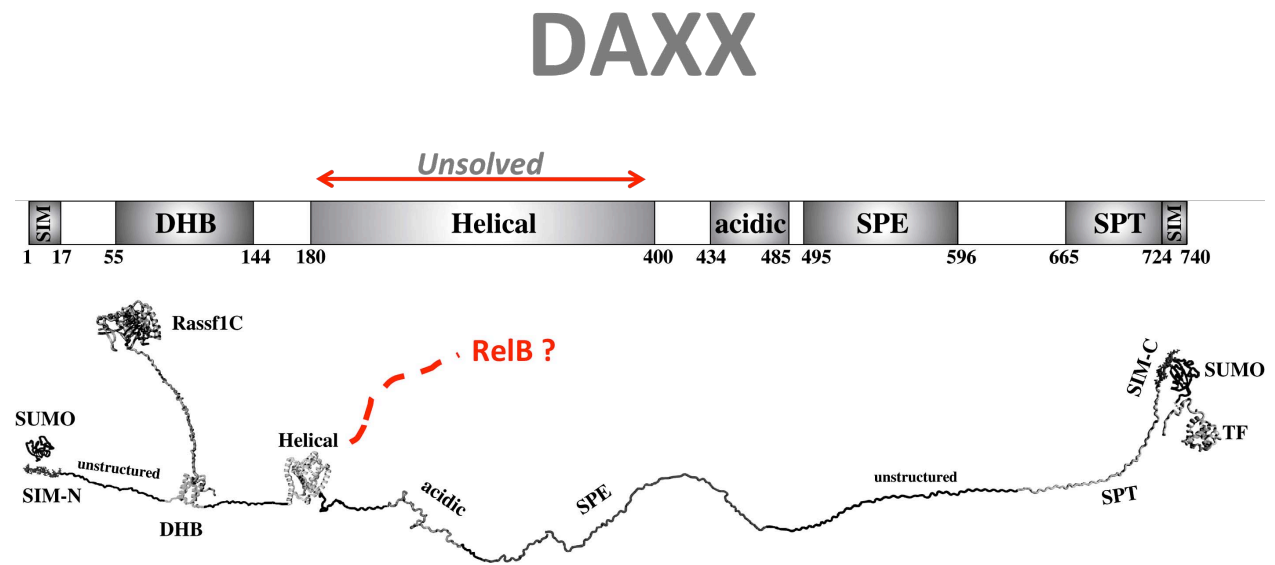
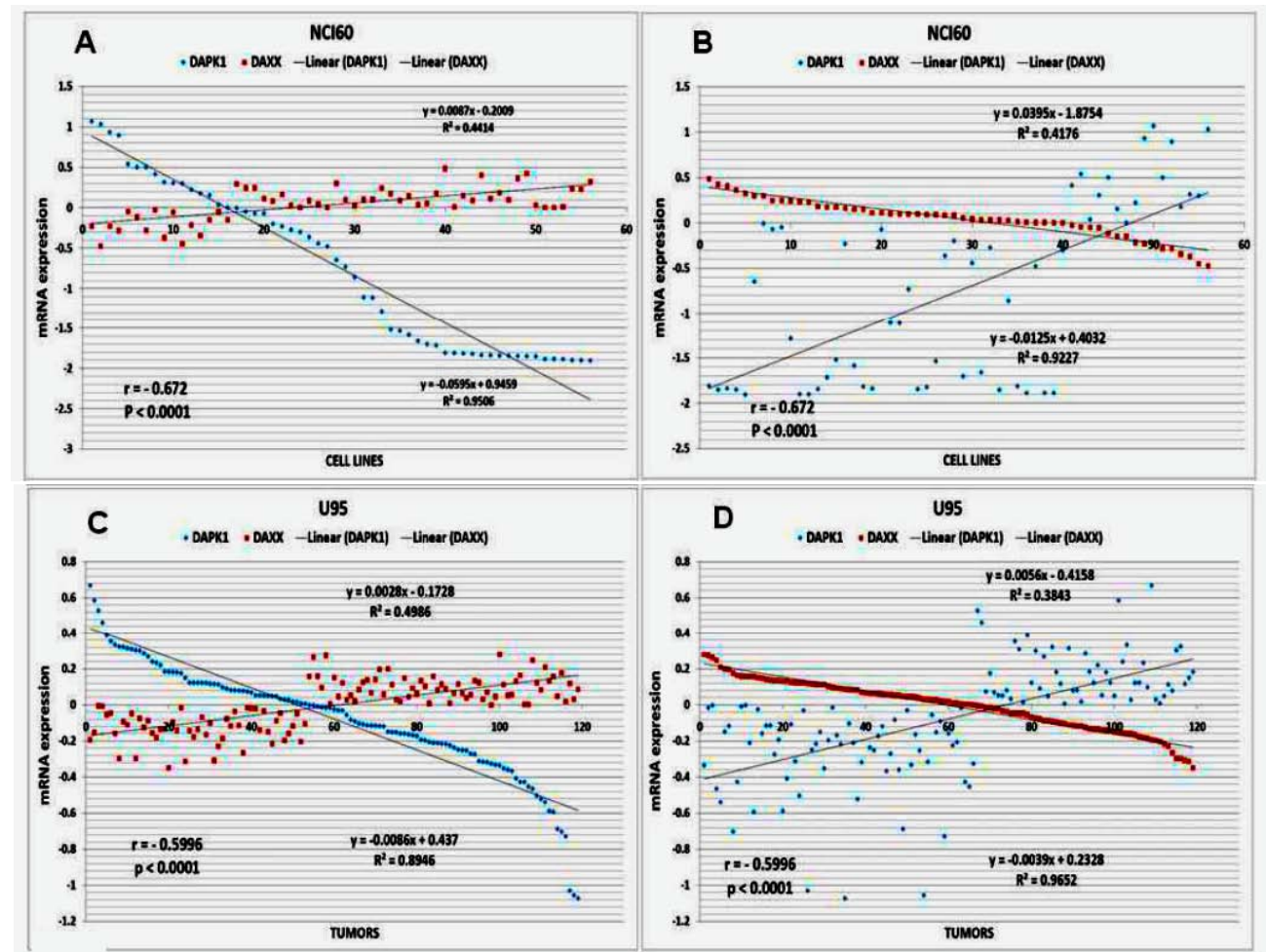


Figure 6



## Key Research Accomplishments

- Through preliminary subcutaneous xenograft experiments, I determined that Daxx is required for tumor growth, because Daxx depletion in ALVA-31 cells correlates with smaller tumors (Figure 1).
- Using shRNA for gene silencing, I successfully silenced Dapk1, Dapk3, and both Daxx and Dapk1 in the prostate cancer cell line ALVA-31 (Figure 2).
- Through preliminary subcutaneous xenograft experiments, I determined that tumor growth rate is faster for DAPK3 K/D and DAXX/DAPK1 double K/D groups, arguing that DAPKs act as tumor suppressors (Figure 2).
- Using purified proteins in pull-down experiments, (1) I established that the interaction between the transcriptional repressor Daxx and the transcription factor RelB is direct (Figure 3); and (2) I identified the respective interacting regions (Figures 3 & 4).
- The affinity tag (GST or His) was removed from both proteins (Daxx and RelB, respectively), and the purified proteins will next be used for attempts at co-crystallization (Figure 5).
- By performing linear regression analysis utilizing publicly available Dapk1 and Daxx mRNA expression data for the NCI60 panel of human cancer cell lines and the U95 collection of human tumors, it was determined that human malignancies display an inverse correlation between Daxx and Dapk1 mRNA expression (Figure 6).

## Reportable Outcomes

This training grant, for the period December 2009 – December 2010, has supported one abstract presentation, which was selected to receive the prestigious AACR award, one middle-author manuscript, and the development of three prostate cancer cell lines with stable knock down of cell death regulatory genes. In addition, based on the work supported by this training grant, I have two pending postdoctoral fellowship applications (submitted to American Cancer Society and Life Sciences Research Foundation) to support my postdoctoral research after the DoD grant expires in December 2011.

The reportable outcomes that have emanated from this grant are summarized below:

### Abstract presentation

Puto, L.A., Kitada, S., Krajewska, M., Glinsky, G., Hunter, T., and Reed, J.C. *Transcriptional repressor Daxx regulates Dapk1 expression, autophagy, apoptosis, and tumorigenicity of prostate cancer cells*. American Association for Cancer Research, Special Conference: Cell Death Mechanisms and Cancer Therapy; February 1-4, 2010; San Diego, California; Abstract # A7.

### Award

02/2010: AACR-AFLAC, Inc. Winner: Scholar-in-Training Award, American Association for Cancer Research, Special Conference: Cell Death Mechanisms and Cancer Therapy, San Diego, CA

### Submitted Manuscript

Brognard, J., Zhang, Y-W., Puto, L.A., and Hunter, T. *Cancer-Associated Loss-of-Function Mutations Implicate DAPK3 as a Tumor Suppressing Kinase*. Revised version submitted to *Cancer Research* in December 2010.

### Development of stable cell lines

- Stable Dapk1 knock-down prostate cancer cell line (ALVA-31 DAPK1 K/D)
- Stable Dapk3 knock-down prostate cancer cell line (ALVA-31 DAPK3 K/D)
- Stable Dapk1 & Daxx double knock-down prostate cancer cell line (ALVA-31 DAPK1/DAXX K/D)

## Conclusion

In the current study, through preliminary subcutaneous xenograft experiments, I have determined that Daxx is required for tumor growth, because Daxx depletion in ALVA-31 prostate cancer (PCa) cells correlates with smaller tumors, and DAPKs act as tumor suppressors, because tumor growth rate is faster for DAPK3 K/D and DAXX/DAPK1 double K/D groups. Moreover, by performing a linear regression analysis of publicly available datasets, an inverse correlation was observed between Daxx and DAPK1 mRNA expression in a diverse collection of human tumor cell lines and tumor specimens, suggesting that Daxx's role as a transcriptional repressor of the DAPK1 gene is broadly relevant to tumor biology, and is not limited to PCa.

In addition, using purified proteins in pull-down experiments, I established that the interaction between Daxx and RelB is direct, and that Daxx amino acids 190-400 are necessary and sufficient for Daxx interaction with RelB. Combined with last year's findings, when we identified the region in RelB (DID-I) responsible for interaction with Daxx, we are now equipped with the necessary information to solve the co-crystal structure of Daxx-RelB. We will then exploit the structure information to probe Daxx's functions.

In summary, our proposed experiments form a structure-function framework for understanding Daxx's functions in vivo. Armed with such information, it may be possible to design peptide inhibitors, delivering them into cells via membrane penetrating sequences, so as to suppress Daxx's tumorigenic actions by blocking RelB-binding sites.

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## Appendices

### **Transcriptional repressor Daxx regulates Dapk1 expression, autophagy, apoptosis, and tumorigenicity of prostate cancer cells**

Lorena A. Puto<sup>1</sup>, Shinichi Kitada<sup>2</sup>, Maryla Krajewska<sup>2</sup>, Gennadi V. Glinsky<sup>3</sup>, Tony Hunter<sup>1</sup>, and John C. Reed<sup>2</sup>

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### **AACR Special Conference: Cell Death Mechanisms and Cancer Therapy**

**February 1-4, 2010; San Diego, California**

Daxx is a transcriptional repressor, binding partner of tumor suppressor PML, and a component of nuclear bodies ("PODs"). Daxx is a modulator of apoptosis sensitivity that silences several apoptosis-regulating RelB target genes through epigenetic mechanisms. The expression of Daxx is altered in many cancers, correlating with changes in sensitivity to chemotherapy. However, the function of Daxx has not been previously determined in prostate cancer cells. In the current study, shRNA was used to silence Daxx in the hormone-refractory prostate cancer (PCa) cell line ALVA-31, investigating the influence of endogenous Daxx on apoptosis gene expression and sensitivity of these tumor cells to apoptotic stimuli. Stable knock-down (K/D) of Daxx using shRNA rendered prostate cancer ALVA-31 cells resistant to cytokine-induced cell death, correlating with increased expression and reduced DNA methylation of RelB target genes *c-FLIP*, *BIRC3*, *DAPK1*, and *DAPK3*. We observed that Daxx deficiency alters expression and DNA methylation of RelB target genes and decreases sensitivity of ALVA-31 cells to apoptosis, thus extending to prostate cancer the mechanisms previously elucidated in MEFs. In subcutaneous tumor xenograft experiments in mice, Daxx K/D resulted in profoundly reduced tumorigenicity of ALVA-31 cells, despite decreased activation of apoptotic caspases. Interestingly, Daxx K/D tumors showed striking changes in markers of autophagy: Beclin-1, LC3, and p62. In culture, Daxx K/D cells also exhibited increased basal and inducible (rapamycin; nutrient deprivation) autophagy. We observed changes in autophagy markers in Daxx K/D cells in culture, including an increase in the LC3-II:LC3-I ratio, decreased p62, and reduced sizes of p62 aggregates when cells were cultured under autophagy-inducing conditions. These alterations in autophagy markers seen in cultured cells are consistent with an increase in autophagy in Daxx K/D subcutaneous tumors, suggesting that Daxx represses the autophagy program. These observations suggest that Daxx has an inhibitory effect on autophagy in vivo. Thus, experimentally ablating Daxx expression appears to promote autophagy. The changes in autophagy markers correlated with marked increases in Daxx K/D cells of expression of Dapk1, a known inducer of autophagy and a target of Daxx-mediated transcriptional repression. Dapk1 protein levels were strikingly elevated in vitro and in vivo in Daxx K/D prostate cancer cells, arguing that Daxx normally helps to reduce levels of this pro-apoptotic and pro-autophagic protein kinase. Furthermore, by using a linear regression analysis of publicly available *Daxx* and *Dapk1* mRNA expression data for the NCI60 panel of human cancer cell lines and the U95 database of human tumor tissue samples (<http://biogps.gnf.org/>), an inverse correlation was found between Daxx and Dapk1 mRNA expression in a diverse collection of human tumor cell lines and tumor specimens, suggesting that Daxx's role as a transcriptional repressor of the *DAPK1* gene is broadly relevant to tumor

biology. Altogether, the findings implicate Daxx in regulation of autophagy, suggesting that Daxx may promote tumorigenesis in some contexts, despite its pro-apoptotic phenotype, possibly by inhibiting autophagy.

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## Appendices, cont.

| CURRICULUM VITAE   |                           |                    |                                    |
|--|---------------------------|--------------------|------------------------------------|
| NAME   |                           | POSITION TITLE     |                                    |
| Puto, Lorena A.  |                           | Research Associate |                                    |
| EDUCATION/TRAINING   |                           |                    |                                    |
| INSTITUTION AND LOCATION   | DEGREE<br>(if applicable) | YEAR(s)            | FIELD OF STUDY                     |
| Point Loma Nazarene University, San Diego, CA                                    | B.A.                      | 1997               | Biology, Chemistry                 |
| University of California, Riverside, CA  | M.S.                      | 2000               | Biochemistry and Molecular Biology |
| Stanford University, Stanford, CA  | Ph.D.                     | 2003-2005          | Immunology                         |
| Sanford-Burnham Medical Research Institute, La Jolla, CA<br>Mentor: John C. Reed |                           | 2005-2009          | Molecular Medicine                 |
| The Salk Institute, La Jolla, CA<br>Mentor: Tony Hunter                          | Research Associate        | 2009-present       | Molecular and Cell Biology         |

## POSITIONS AND HONORS:

### Positions and Employment

01/1999-06/1999: Teaching Assistant, Biochemistry 162 (Biochemistry and Molecular Biology Laboratory) and Biochemistry 110B (General Biochemistry), University of California, Riverside, CA

09/2000-08/2003: Research Assistant, Dr. Gary Bokoch, The Scripps Research Institute, La Jolla, CA

09/2003-08/2005: Ph.D. Student, Biological Sciences & Immunology, Stanford University, Stanford, CA

04/2004-06/2004: Teaching Assistant, Biological Sciences 129 (Cellular Dynamics), Stanford University, Stanford, CA

09/2005-10/2009: Ph.D. Candidate, Dr. John C. Reed, Molecular Medicine, Sanford-Burnham Medical Research Institute, La Jolla, CA

11/2009-present: Postdoctoral Fellow, Dr. Tony Hunter, Molecular and Cell Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA

#### Honors, Awards and Memberships (selected)

- |              |   |
|--------------|---|
| 2003         | F1000 Most Recommended: Faculty of 1000 Biology: Puto, L.A., et al. J Biol Chem, 2003.  |
| 2003-2004    | Cellular and Molecular Biology (CMB) Training Grant, Stanford University  |
| 2007-2008    | Dissertation Award, California Breast Cancer Research Program   |
| 2007-present | Member, American Association for Cancer Research  |
| 2008         | AACR-AFLAC, Inc. Scholar-in-Training Award, 99th Annual Meeting, American Association for Cancer Research, San Diego, CA  |
| 2008-present | Dissertation Award, Department of Defense   |
| 2009         | AACR-AFLAC, Inc. Scholar-in-Training Award, American Association for Cancer Research, Special Conference: Advances in Prostate Cancer Research, San Diego, CA     |
| 2010         | AACR-AFLAC, Inc. Scholar-in-Training Award, American Association for Cancer Research, Special Conference: Cell Death Mechanisms and Cancer Therapy, San Diego, CA |

#### **PUBLICATIONS :**

##### Research Papers

1. Puto, L.A., Pestonjamas, K., King, C.C. and Bokoch, G.M. (2003) p21-activated kinase 1 (PAK1) interacts with the Grb2 adapter protein to couple to growth factor signaling. J Biol Chem 278:9388-9393.
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4. Puto, L.A., Kitada, S., Krajewska, M., Glinsky, G.V., Colon, J., Baker, C.H., and Reed, J.C. (Under revision) Transcriptional repressor Daxx regulates Dapk1 expression, autophagy, apoptosis, and tumorigenicity of prostate cancer cells.
5. Brognard, J., Zhang, Y-W., Puto, L.A., and Hunter, T. Cancer-Associated Loss-of-Function Mutations Implicate DAPK3 as a Tumor Suppressing Kinase. Revised version submitted to *Cancer Research* in December 2010.

#### Abstracts

1. Puto, L.A. and Reed, J.C. Daxx controls methylation of RelB target genes involved in apoptosis regulation. Proceedings of the 99th Annual Meeting of the American Association for Cancer Research; April 12-16, 2008; San Diego, CA; Oral Presentation, Abstract # 2534.
2. Puto, L.A. and Reed, J.C. Role of Daxx in Regulating Apoptosis of Prostate Cancer. American Association for Cancer Research, Special Conference: Advances in Prostate Cancer Research; January 21–24, 2009; San Diego, CA, Abstract # A19.
3. Puto, L.A. and Reed, J.C. Epigenetics mechanisms of Daxx in Prostate Cancer. Gordon Research Conference on Cancer Genetics and Epigenetics; January 25-30, 2009, Ventura, CA; Abstract # 32.
4. Puto, L.A., Kitada, S., Krajewska, M., Glinsky, G., Hunter, T., and Reed, J.C. Transcriptional repressor Daxx regulates Dapk1 expression, autophagy, apoptosis, and tumorigenicity of prostate cancer cells. American Association for Cancer Research, Special Conference: Cell Death Mechanisms and Cancer Therapy; February 1-4, 2010, San Diego, CA; Abstract # A7.